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2,3-Bisphosphoglycerate Inhibits Hemoglobin Synthesis and Phosphorylation of Initiation Factor 2 by Casein Kinase II in Reticulocyte Lysates

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2,3-Bisphosphoglycerate inhibited protein synthesis in reticulocyte lysates with 50% inhibition at 2 mM. Glycerate 2,3-P_2 increased the Mg^{2+} optimum for protein synthesis by chelation of Mg^{2+}, but Mg^{2+} addition did not completely reverse the inhibition, suggesting an additional site of action. eIF-2 has been used to examine the activity of casein kinase II in reticulocyte lysates in response to glycerate 2,3-P_2. When glycerate 2,3-P_2 was increased to 4mM, phosphorylation of eIF-2β was increasingly inhibited. Thus inhibition of phosphorylation of translational components by casein kinase II can be correlated with inhibition of globin synthesis at physiological concentrations of glycerate 2,3-P_2.

2,3-Bisphosphoglycerate is one of the most abundant phosphorylated metabolites in red blood cells. The concentration of glycerate 2,3-P_2 changes during development and in a number of physiological and pathological conditions (1). The concentration is approximately equal to that of hemoglobin, and glycerate 2,3-P_2 binds preferentially to the deoxy form, altering the equilibrium between oxygenated and deoxygenated hemoglobin. The concen-

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ABBREVIATIONS: glycerate 2,3-P_2, 2,3-bisphosphoglycerate; glycerate 3-P, 3-phosphoglycerate; eIF-, eukaryotic initiation factor.

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tration of free glycerate 2,3-P_2 is estimated to be 1-3 mM in rabbit reticulocytes (2) with a total concentration of 8-10 mM (3,4). The concentration of glycerate 2,3-P_2 rises as reticulocytes mature into erythrocytes (5), coincident with the loss of protein synthesis.

Glycerate 2,3-P_2 inhibits purified casein kinase II at physiological concentrations with both casein and initiation factors as substrate (2,6-8). Casein kinase II phosphorylates a number of proteins involved in protein synthesis including initiation factors 2, 2B, 3, 4B and 5 (2,9-11) and messenger ribonucleoprotein particle proteins (12). Thus, we examined the effects of addition of glycerate 2,3-P_2 to reticulocyte lysates on phosphorylation of eIF-2 by casein kinase II and on protein synthesis. eIF-2 forms a ternary complex with met-$tRNA_i$ and GTP which binds to the 40S subunit to form a preinitiation complex and is one of the initial steps in the initiation process (13,14).

EXPERIMENTAL PROCEDURES

Materials. Glycerate 2,3-P_2 (pentacyclohexylammonium or Tris salt) and glycerate 3-P were obtained from Sigma. [^{32}P]Orthophosphate and [^{14}C]leucine were from ICN. [γ-^{32}P]ATP was prepared as described (10). Heme (equine) was obtained from Schwarz/Mann and prepared (15) just prior to use. Highly purified casein kinase II (16) was generously provided by Dr. Gary M. Hathaway. eIF-2 was purified from rabbit reticulocytes as described by Merrick (17) with minor modifications.

In vitro protein synthesis. Reticulocyte lysate was prepared from phenylhydrazine-treated rabbits (18) and stored at -70°C. Protein synthesis was measured by incorporation of [^{14}C]leucine into protein (18). Glycerate 2,3-P_2 and glycerate 3-P were prepared in distilled water and the pH was adjusted to 7.4. Hemin, glycerate 2,3-P_2, and glycerate 3-P were added as indicated; incubation was at 34°C for 20 min. Reactions were terminated by precipitation with 10% trichloroacetic acid (18).

Phosphorylation of eIF-2 in reticulocyte lysate. [^{32}P]Orthophosphate (5.6 mCi) was equilibrated with the γ-phosphate of ATP by incubation with 0.4 ml of the phosphate exchange-protein synthesizing mixture for 45 min at 34°C as described previously (18). Aliquots (0.020 ml) were added to freshly thawed lysate (0.035 ml) and incubated with hemin (25 μM) and/or glycerate 2,3-P_2 for 20 min. Reactions were terminated with sample buffer (0.025 ml) and aliquots (0.005 ml) were analyzed by SDS-polyacrylamide gel electrophoresis (10). Purified eIF-2 (5 μg) phosphorylated by casein kinase II (150 units) (7) was used as a reference. The radiolabel was visualized by autoradiography and quantified by densitometric scanning (10).

RESULTS

Inhibition of protein synthesis by glycerate 2,3-P_2. The effect of glycerate 2,3-P_2 on protein synthesis was examined in a

Table I. Inhibition of Protein Synthesis by Glycerate 2,3-P_2*

Conditions	(^{14}C)Leucine Incorporated (cpm)	Inhibition (%)
Control	21,497	0
- Hemin	4,893	77
+ Glycerate 2,3-P_2 (1 mM)	18,676	13
(2 mM)	10,552	51
(4 mM)	2,136	90
+ Glycerate 3-P (4 mM)	14,941	31

* Protein synthesis was assayed as described in Experimental Procedures with glycerate 2,3-P_2 or glycerate 3-P as indicated. All assays contained 25 μM hemin unless otherwise indicated.

cell-free protein synthesizing system from reticulocytes. Incorporation of [^{14}C]leucine into protein was measured with 25 μM hemin and 2.0 mM $MgCl_2$ in the presence and absence of glycerate 2,3-P_2 (Table I). Increasing inhibition of protein synthesis was observed at increasing concentrations of glycerate 2,3-P_2; 50% inhibition was observed at 2 mM and 90% inhibition at 4 mM. In contrast, 31% inhibition was observed at 4 mM glycerate 3-P. When hemin was omitted, protein synthesis was inhibited by 77%. Incorporation of [^{14}C]leucine into hemoglobin in a hemin-supplemented lysate was linear and not biphasic in the presence and absence of glycerate 2,3-P_2. However, in the presence of glycerate 2,3-P_2, the rate of protein synthesis was significantly reduced (Table I).

Effect of glycerate 2,3-P_2 on the Mg^{2+} optimum for hemoglobin synthesis. The Mg^{2+} optimum for hemoglobin synthesis was examined at increasing concentrations of glycerate 2,3-P_2 (Figure 1). In the absence of glycerate 2,3-P_2, 1 mM Mg^{2+} was optimal for protein synthesis. Upon addition of glycerate 2,3-P_2, inhibition of protein synthesis was accompanied by a shift in the optimal Mg^{2+} concentration from 1 to 3 mM. This shift was due in part to chelation of Mg^{2+} ions by the negatively charged organophosphate. However, a significant portion of the inhibition could not be reversed by increasing concentrations of Mg^{2+}.

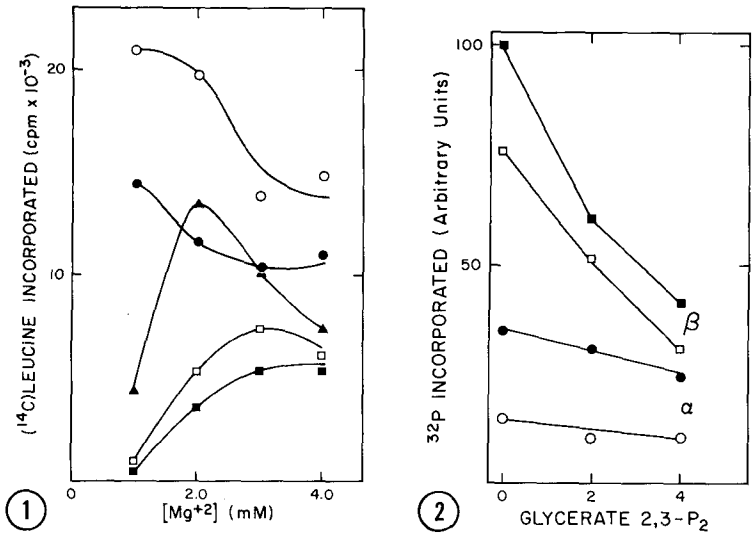

Figure 1. Mg^{2+} optimum for protein synthesis in the reticulocyte lysate at increasing concentrations of glycerate 2,3-P_2.

Protein synthesis was carried out with 25 μM hemin and 1-4 mM Mg^{2+} in the absence (○–○), or presence of glycerate 2,3-P_2 at 1 mM (●–●), 2 mM (▲–▲), 3 mM (□–□), and 4 mM (■–■).

Figure 2. Phosphorylation of eIF-2 in reticulocyte lysate.

Phosphorylation of eIF-2 was analyzed by gel electrophoresis and ^{32}P incorporated into eIF-2β was quantified by densitometric scanning of the autoradiogram. Phosphorylation of eIF-2β in the hemin-deprived lysate in the absence of glycerate 2,3-P_2 was set at 100%. eIF-2β in the absence of hemin (■–■); eIF-2β with 25 μM hemin (□–□); eIF-2α in the absence of hemin (●–●); eIF-2α in the presence of hemin (○–○).

Phosphorylation of eIF-2 in reticulocyte lysates. In order to examine whether the inhibitory effect of glycerate 2,3-P_2 on hemoglobin synthesis was correlated with a decrease in phosphorylation of translational components by casein kinase II, the phosphate exchange-protein synthesizing system of Floyd and Traugh (18) was used to maintain ATP and GTP at constant specific activity in the lysate during protein synthesis. Following incubation, the 0.5 M KCl salt wash fraction was isolated from polysomes and analyzed. Identification of the β subunit of eIF-2 (M_r 55,000) was based on comigration on polyacrylamide gels with purified eIF-2 phosphorylated by casein kinase II. As shown in Figure 2, 40% inhibition of phosphorylation of eIF-2β was observed at 2 mM glycerate 2,3-P_2 in the hemin-supplemented lysate and 60% inhibition at 4 mM. Significant inhibition of phosphorylation of some of the other components in the 0.5 M KCl salt wash

by glycerate 2,3-P_2 was also observed (data not shown). Phosphorylation of eIF-2α by the hemin controlled repressor was significantly inhibited by 25 μM hemin, but was not affected by glycerate 2,3-P_2 (Figure 2). Hemin had little effect on phosphorylation of eIF-2β.

DISCUSSION

To probe the relationship between phosphorylation of translational components by casein kinase II and protein synthesis, we utilized a physiological effector of casein kinase II, glycerate 2,3-P_2 (8). This compound acts as a competitive inhibitor of casein kinase II with respect to eIF-2, with an apparent inhibitory constant of 1 mM (7). Significant inhibition of protein synthesis is observed when glycerate 2,3-P_2 is added to the lysate. The inhibition is concentration-dependent and is observed at all concentrations of Mg^{2+} (1-4 mM) tested. Addition of glycerate 2,3-P_2 causes a shift in the Mg^{2+} optimum for protein synthesis and a marked inhibition of globin synthesis. The inhibition is not totally reversible by the addition of Mg^{2+}. Thus, the inhibitory effect is not due merely to complexation of Mg^{2+} ions.

Regulation of protein synthesis by glycerate 2,3-P_2 using gel-filtered lysate has been reported previously by Narita et al. (19). These authors found that low concentrations of glycerate 2,3-P_2 (up to 1 mM), markedly stimulated protein synthesis at Mg^{2+} concentrations of 1.5-4.0 mM, while glycerate 2,3-P_2 higher than 1 mM inhibited protein synthesis. When we examined gel-filtered lysate, the results were almost identical to those presented herein, with only an inhibition of protein synthesis observed (data not shown).

The inhibition of protein synthesis by glycerate 2,3-P_2 is accompanied by inhibition of phosphorylation of eIF-2β as well as other components in the 0.5 M KCl wash. This is due to direct inhibition of casein kinase II by glycerate 2,3-P_2 (7,8). In these studies, eIF-2 is used as a marker; since casein kinase II phosphorylates at least 4 other initiation factors (2,9-11), it would be expected that some of these would coincide with those proteins in the 0.5 M KCl wash whose phosphorylation is inhibited by glycerate 2,3-P_2.

A large number of proteins, from membrane to nucleus, are phosphorylated by casein kinase II. Hathaway and Traugh (2) have proposed that casein kinase II functions to integrate total cell

metabolism and active enzyme is required for maintenance of cell viability and normal physiological activity. These studies substantiate this premise. Recently, Dholakia and Wahba (11) have shown that the largest subunit of eIF-2B (guanine nucleotide exchange factor) is phosphorylated by casein kinase II. The enhanced phosphorylation of eIF-2B stimulates the exchange of GDP associated with eIF-2 following one round of translation, for GTP. This would have a stimulatory effect on hemoglobin synthesis. Coordinate regulation of phosphorylation of eIF-2β, eIF-2B, as well as other initiation factors, by casein kinase II, may be responsible for the inhibition of protein synthesis observed with glycerate 2,3-P_2.

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